

## Phytochemical, Antioxidant and Antibacterial Activity of Black Tea (*Camellia Sinensis*)

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### ABSTRACT

The aim of present study to evaluated the phytochemical composition, antioxidant and antibacterial activities of commercially available black tea (*Camellia sinensis*). Methanol, ethyl acetate and acetone extracts black tea were prepared by soxhlet extraction. Antioxidant activity was performed by DPPH free radical scavenging assay, reducing power assay and total antioxidant capacity assay. The antimicrobial activity was analyzed for Gram positive bacteria *Bacillus cereus* ATCC13061, *Staphylococcus aureus* ATCC6538p, *Staphylococcus saprophyticus* KCTC3345, *Listeria monocytogenes* ATCC7644 and Gram negative bacteria *Proteus vulgaris* KCTC2512, *Pseudomonas aeruginosa* KCTC2004, *Pseudomonas putida* ATCC49128 and *Serratia marcescens* KCTC42171. Phytochemical results indicated presence of flavonoids, tannins, triterpenes, lipid and reducing sugars. The methanol extract exhibits strong antioxidant activity and all bacteria were susceptible to the methanol extract. The results demonstrated that black tea has antioxidant activity and antimicrobial activity and importance as an alternative antioxidant and antimicrobial agent in therapeutics and food industry.

**Key words:** Antibacterial, Antioxidant, Black tea, *Camellia sinensis*, Food pathogens.

### INTRODUCTION

The natural antioxidants can prevent human body from toxic effect of free radicals and chronic diseases as well as retarding lipid oxidative rancidity in food. Synthetic antioxidants are harmful due to toxicity and carcinogenicity. The use of synthetic antioxidants and preservatives is being questioned in food industry. Therefore, there is necessity to replace natural product as an antioxidant as well as antibacterial instead of synthetic agents. The prior need of life is safe and secure health, throughout the globe variety of bacterial species getting resistance to antibiotics<sup>1</sup> and re-emerging infectious agents need to discover alternative compounds with new mechanism of action and with diverse chemical structure. At the present time, search for new product with multifunctional property such as antioxidant and antimicrobial is a very active domain of research. The plants produce secondary metabolites to protect themselves from foreign agents<sup>2</sup>. So, the plant extracts are the best and natural source for finding new compounds with novel mechanisms of action against bacteria as well as with antioxidant activity. *Camellia sinensis* plant leaves are used in production of different type of tea (Green, Black, White and Oolong Tea) and the plants are grown best in certain tropical and subtropical regions<sup>3</sup>. The tea drink is famous in Asian countries, in India called Chai,

particularly in Indian tradition the black tea powder is boiled in water with addition of sugar and milk for good taste and drinking every day. In traditional herbal medicines, a black tea boiled with water been used as a medicine for stomach pain and dysentery. Drinking tea also effects on attention and mood<sup>4</sup>. Tea from *C. sinensis* has many medicinal properties as well as health benefits. Medicinal properties of tea include anticancer<sup>5</sup>, anti-inflammatory effect<sup>6-8</sup>, antioxidant<sup>9</sup>, antiviral<sup>10</sup>, anti-helminthes<sup>11</sup> and antimicrobial<sup>12,13</sup>. Usage of natural product as an antioxidant or antimicrobial agent is safe and better for health but its applications are limited due to only little research work till date. Foods born diseases are considered to be a major health threat in the world wide. Packaged food material is one of the source for pathogenic bacteria<sup>14</sup> and bacteria can grow well due to chemical diversity of meat provides suitable conditions for growth<sup>15</sup>. In this study we focused on define the preliminary phytochemical composition, antioxidant activity and antibacterial effect of commercially available *C. sinensis* black tea. This is the first reporting of *C. sinensis* black tea methanol extract study for not only antioxidant activity but also antibacterial activity against food related pathogenic and putrefactive bacteria.

### MATERIALS AND METHODS

Table 1: Phytochemical analysis

Phytochemical tests	Solvents		
	Methanol	Acetone	Ethyl acetate
Flavonoids	+	-	-
Tannins	+	+	+
Triterpenes	+	-	-
Lipids	+	+	+
Reducing sugars	+	-	-
Amino acids	-	-	-
Polysaccharides	-	-	-
Steroids	-	-	-

+: Present, -: Absent.

**Materials:** Solvents: Methanol, Ethyl Acetate and Acetone were purchased from Sigma Aldrich, St. Louis, USA. **Chemicals:** DMSO (Dimethyl sulfoxide), DPPH (2,2-Diphenyl 1-1-Pikryl-hydrazyl) and Ascorbic acid were obtained from Sigma Aldrich, USA. Potassium ferricyanide, Tri-chloroacetic acid, Ferric chloride and Ammonium molybdate were procured from Daejung Chemicals & Metals Co., Gyeonggi-do, Korea. **Media:** Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were obtained from BD Diagnostic, Le Pont de Claix, France.

**Plant material and extraction:** The commercially available black tea with brand name Red Label® was purchased from supermarket at Mumbai, India. Thimble was filled with 20 g of tea sample for each solvent and extracted using soxhlet apparatus (Pyrex soxhlet extraction apparatus, capacity 500 ml, to top of siphon). The tea material was successively extracted with 200 ml of methanol for 24 hours. Same procedure applied for other solvents, Ethyl Acetate and Acetone. The resulting extract of methanol (TM), ethyl acetate (TEA) and acetone (TA) was evaporated in rotary evaporator apparatus (Heidolph, Mod No. IM R 1103-035) at 50°C, 45°C and 40°C respectively, residue was stored at -20°C until requirement. Stock solutions of 100 mg/ml were prepared by dissolving extract residue in dimethyl sulfoxide (DMSO) and were sterilized by filtration through syringe membrane filter 0.2 µm (Minisart®, Sartorius Stedim Biotech, GmbH, Germany). The stock solution was stored at 4°C for further usage.

**Phytochemical Study:** Phytochemical analysis was performed for preliminary phytochemical composition in freshly prepared extract solutions using the methods with modifications<sup>16,17</sup>. **Test for flavonoids:** Test sample was dissolve in ethanol, warmed and solution were filtered then magnesium metal chips were added to the filtrate followed by few drops of conc. HCl. Formation of red to purple or pink, orange color in solution indicated the presence of flavonoids. **Test for tannins:** About 0.25 g of sample was vortexed in 5.0 ml of distilled water and then filtered. To 2.0 ml of the filtrate few drops of 1% FeCl<sub>3</sub> were added. Formation of green, blue-green or blue-black color in solution indicated the presence of tannins. **Test for**

**triterpenes:** To the test solution CHCl<sub>3</sub>, conc. H<sub>2</sub>SO<sub>4</sub> was added. Formation of red color in solution indicated the presence of triterpenes. **Test for lipids:** Iodine solution was added dropwise to the test solution. Disappearance of iodine original color in solution indicated the presence of lipids. **Test for reducing sugars:** Equal volume of Fehling's solutions A and B were added to make the test solution and kept in boiling water bath for 2-3 min. Formation of brick red color precipitates in solution indicated the presence of reducing sugars. **Test for protein (Free amino acids):** 2-4 drops of ninhydrin were added on test solution and kept in boiling water bath for 2-3 min. A purple color solution formation indicated the presence of free amino acids. **Test for polysaccharides:** Few drops of iodine solution were added to test solution. Formation of blue color solution indicated the presence of polysaccharides. **Test for steroids:** About 1.0 ml of test solution was mixed vigorously with 1.0 ml of conc. H<sub>2</sub>SO<sub>4</sub>. Steroids and H<sub>2</sub>SO<sub>4</sub> layers gets separated and formation of test solution layer with cherry red color and acid layer with green color indicated the presence of steroids.

#### Antioxidant assay

**DPPH free radical scavenging assay:** The capacity to scavenge the "stable" free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed based on method described by Tian et al<sup>18</sup> and Ferreira et al<sup>19</sup>. Briefly, 1 ml of TM extract with different concentration (10, 20, 30, 40, 50 and 60 µg/ml) in methanol was added to 4 ml of methanol contained 0.07 mM DPPH. The reaction mixture was shaken vigorously and incubated at room temperature for 30 min in dark. The DPPH radical reaction was monitored by recording absorbance at 517 nm. Methanol and Ascorbic acid were used as blank and standard respectively. The decreased absorbance value of reaction mixture indicated an increased percentage of free radical scavenging activity. The percentage inhibition or free radical scavenging activity was calculated by using the following formula;

$$\% \text{ Inhibition} = \frac{[(\text{Control absorbance} - \text{Test absorbance}) / \text{Control absorbance}] \times 100}{100}$$

Where, the control was contained only methanol and DPPH solution. The IC<sub>50</sub> value (µg/ml) is the concentration required to inhibit 50% of the initial DPPH free radical, was calculated from the graph of inhibition curve. All the reactions were monitored in triplicate and the value were expressed as the mean ± standard deviation (S.D.).

**Reducing power assay:** Reducing power assay was performed on the bases of procedure have been reported<sup>19,20</sup>. Briefly, 1 ml of each different concentration of TM extract (50, 100, 150, 200 and 250 µg/ml) were added to mixture containing 2 ml of phosphate buffer (0.2M, pH6.6) and 2 ml of 1% Potassium ferricyanide. The mixture were shaken and incubated in water bath at 50°C for 20 min. After that, 2 ml of 10% Tri-chloroacetic acid (w/v) were added and the mixture was centrifuged at 3,000 rpm for 10 min. Finally 2 ml of upper layer solution was diluted with equal volume of distilled water followed by adding 0.5 ml of 0.1% Ferric chloride and then the absorbance was measured at 700 nm. Increased absorbance

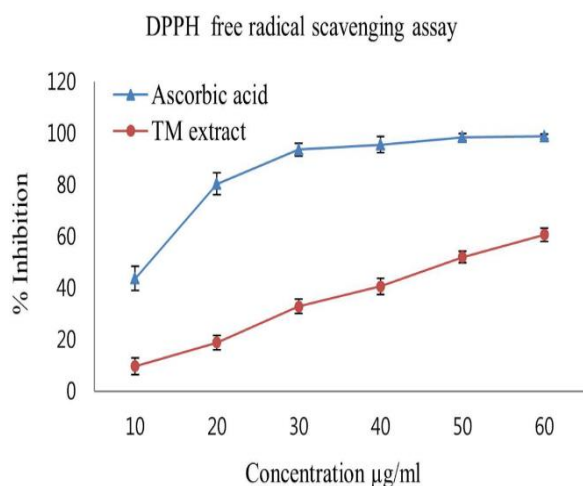


Figure 1: DPPH free radical scavenging assay for TM and Ascorbic acid. Error bar indicates mean of triplicates  $\pm$  standard deviation.

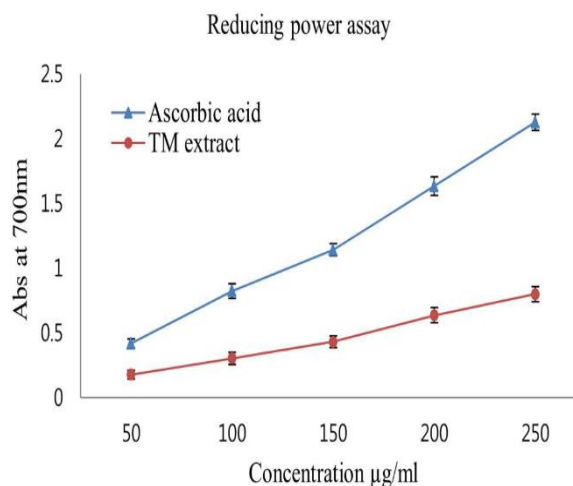


Figure 2: Reducing power assay for TM and Ascorbic acid. Data were expressed in mean of triplicates  $\pm$  standard deviation.

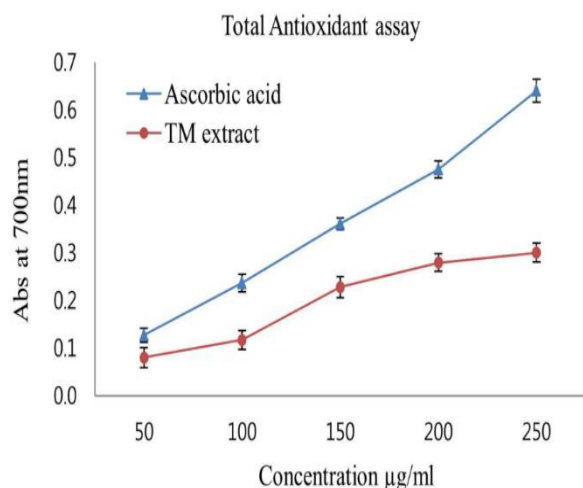


Figure 3: Total antioxidant assay. Total antioxidant assay of TM and Ascorbic acid. Measurements were presented in mean of triplicates  $\pm$  standard deviation

value indicated higher the reducing power. Ascorbic acid was used as a standard and phosphate buffer as a blank solution.  $IC_{50}$  is the value of sample exact concentration which provides absorbance 0.5 at 700 nm. The assay was performed in triplicates and the results expressed as mean  $\pm$  standard deviation (S.D.).

**Total antioxidant capacity assay:** Total antioxidant capacity assay was performed by following methods described by Manivasagan et al<sup>20</sup> and Prabhu et al<sup>21</sup>. The TM extract, 0.3 ml of different concentration (50, 100, 150, 200, and 250  $\mu$ g/ml) were added on screw cap test tube containing 3.0 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate in 1:1:1 ratio). The capped tubes were incubated in a water bath at 90°C for 90 min. After those solutions were allowed to cool at room temperature and absorbance measured at 700 nm against blank (without the extract). Ascorbic acid was used as a standard. The total antioxidant capacity was expressed as equivalent of ascorbic acid. The exact concentrations of sample at absorbance 0.5 were expressed as  $IC_{50}$  value. The assay was repeated three

times and the results are expressed as mean  $\pm$  standard deviation (S.D.).

#### Antibacterial activity

**Microorganisms:** The pathogenic bacteria used in the study were purchased from Korean Culture Center of Microorganisms (KCCM), Republic of Korea. Specific strains used were Gram positive bacteria *Bacillus cereus* ATCC13061, *Staphylococcus aureus* ATCC6538p, *Staphylococcus saprophyticus* KCTC3345, *Listeria monocytogenes* ATCC7644 and Gram negative bacteria *Proteus vulgaris* KCTC2512, *Pseudomonas aeruginosa* KCTC2004, *Pseudomonas putida* ATCC49128, and *Serratia marcescens* KCTC42171. All bacterial strains were maintained on Tryptic Soy Agar plates (TSA) at 4°C upto 3 months and stocks were preserved at -80°C in 30% glycerol containing Tryptic Soy Broth medium (TSB).

**Inoculum preparation:** Initially, bacterial isolated colonies were obtained by streaking on TSA plate and incubated at 37°C for 24 hours. Selected colonies were resuspended in sterile normal saline solution to standardize culture turbidity absorbance from 0.08 to 0.1 at 625 nm (equal to 0.5 McFarland) was obtained by spectrophotometry<sup>22</sup> (Libra S22, Biochrom Ltd., Cambridge, England).

**Disc-diffusion assay:** Antibacterial susceptibility test using disc diffusion method developed by Kirby and Bauer and previously described<sup>23, 24</sup> were used with some modification. Briefly, Mueller Hinton Agar (MHA) plates were spread with 100  $\mu$ l of standardized bacterial suspension and spread over with flame sterilized glass rod. 50  $\mu$ l of TM, TEA and TA stock solution (100 mg/ml) was added on standard size 8 mm filter disc (Advantec, Toyo Roshi, Japan). Discs were air dried and placed on agar surface, only DMSO was used as a negative control and Ampicillin 100  $\mu$ g/ml (Sigma Aldrich Chemie, GmbH, Germany) as a positive control. All plates were incubated at 37°C for 24 hours. After incubation period zone of inhibition (ZOI) was measured in mm.

**Minimum Inhibitory Concentration (MIC):** After determination of antimicrobial activity of the extracts

Table 2: Antibacterial activity and MIC

Microorganisms	ZOI in mm*		MIC <sup>a</sup> Mg/ml
	TM 100mg/ml	Ampicillin 100mg/ml	
<i>B. cereus</i> ATCC 13061	16.67±0.58	13.33±0.58	3
<i>S. saprophyticus</i> KCTC3345	16.00±1.00	19.33±0.58	9
<i>S. aureus</i> ATCC6538p	14.67±1.15	22.33±1.53	9
<i>L. monocytogenes</i> ATCC7644	14.33±0.58	17.33±0.58	10
<i>P. vulgaris</i> KCTC2512	17.33±0.58	15.67±0.58	5
<i>P. aeruginosa</i> KCTC2004	12.33±0.58	ND	13
<i>P. putida</i> ATCC49128	15.00±1.00	23.00±1.00	8
<i>S. marcescens</i> KCTC42171	13.33±1.15	ND	10

MIC<sup>a</sup>: MIC for TM extract, ND: Not detected. Data represented as mean of triplicates ± standard deviation.

against test pathogens, MIC assay was performed as per method describe previously<sup>22, 25</sup> with slight modifications. Briefly, different dilutions (mg/ml) of TM were prepared in DMSO. 50 µl of each dilution were dropped on a sterile paper disc with the help of sterile micropipette tips and air dry. The discs were kept on MHA agar plate seeded with 100 µl of standardized culture suspension (absorbance from 0.08 to 0.1 at 625 nm) separately and the plates were incubated at 37°C for 24 hours. DMSO only was used as a negative control. The experiments were carried out under Laminar Air Flow Cabin (Jeio Tech, Daejeon, Korea). After incubation MIC was determined as the lowest concentration of extract were showed zone of inhibition. Whole experiment was repeated in triplicate. TEA and TA was not analyzed due to very less yield and any significant results were not observed in phytochemical study.

Data analysis: All data was measured average values of triplicates. The results were subjected to Microsoft excel 2007 and are expressed as mean ± standard deviation (S.D.).

## RESULTS AND DISCUSSION

**Phytochemical study** The results were shown presence of flavonoids, triterpenes, tannins, lipid and reducing sugar in TM, while tannins and lipids were only present in TEA and TA. Amino acids, polysaccharides and steroids were absent on all the extracts. Methanol solvent contains various phytochemicals compared with acetone and ethyl acetate (Table 1). TEA and TA was not further analyzed for antioxidant activity and for antimicrobial activity (data not shown) due to very less and no significant results were observed in flavonoids, triterpenes, and reducing sugar. **Antioxidant activity** As single method cannot provide ability of compound for their antioxidant ability, in the study we used three different methods such as DPPH free radical scavenging assay, reducing power assay and total antioxidant assay. **DPPH free radical scavenging assay** Radical scavenging activity was performed by using *in vitro* DPPH free radical model. When DPPH radicals encounter a proton donating substance such as an antioxidant, the radical is scavenged and absorbance is decreased by changing color from purple to yellow<sup>20, 26</sup>. The reducing activity of TM and ascorbic acid (as a standard) was measured spectrophotometrically and 50% inhibition of DPPH radical scavenging activity were expressed as IC<sub>50</sub> (Fig. 1). TM showed good DPPH radical

scavenging activity in a dose dependent manner. Our results were complies with the previous results reported by Ferreira et al<sup>19</sup> and Manivasagan et al<sup>20</sup> who reported that the DPPH scavenging activity increased with increasing concentration. For DPPH radicals the IC<sub>50</sub> values were recorded to be 48.67±1.53 µg/ml and 11.67±0.58 µg/ml for TM and ascorbic acid respectively. The literature review revealed that the antioxidant activity of the methanolic extract of commercially available *C. sinensis* black tea has not been previously reported. **Reducing power assay** The antioxidant (reducers) causes the reduction of Fe<sup>3+</sup>/ferricyanide complex to the ferrous form. The Fe<sup>2+</sup> complex can be monitored by measuring the formation of Perl's Prussian blue at 700nm. As shown in Fig. 2 TM extract exhibited good reducing power in a dose dependent manner. The IC<sub>50</sub> value is the exact concentration for absorbance 0.5 at 700 nm. The IC<sub>50</sub> of TM was 167.33±0.58 µg/ml while ascorbic acid (standard) showed 55.67±0.58 µg/ml. The results indicated as the concentration increases the reducing power also increases and it was similar to previously reported results of reducing power in Ferreira et al<sup>19</sup> and Manivasagan et al<sup>20</sup>. The dose dependent graph of TM reducing activity was lower compare to ascorbic acid as a standard. **Total antioxidant assay** The antioxidant assay is based on reduction of Mo(VI) to Mo(V) by the reducers (antioxidants) and formation of a green phosphate/Mo(V) complex with a absorbance maxima at 700 nm<sup>21</sup>. The assay was used to evaluate total antioxidant activity of TM extract and ascorbic acid (standard). The results showed in Fig. 3 indicated dose dependent increasing value of total antioxidant activity. The IC<sub>50</sub> value expressed as an exact concentration provided absorbance 0.5 at 700 nm. The IC<sub>50</sub> for TM extract and ascorbic acid were recorded as 412.67±0.58 µg/ml and 212.0±1.0 µg/ml respectively. Our findings were complies with the results of Manivasagan et al<sup>20</sup> and Prabhu et al<sup>21</sup> for dose dependent increase of total antioxidant activity. **Antibacterial activity** The antibacterial activity of TM, TEA and TA extracts of *C. sinensis* black tea was initially performed using disc diffusion assay. Extract 50 µl of 100 mg/ml stock solution were tested on all selected bacteria together with Ampicillin 100 µg/ml as a positive control and 100% DMSO, the extract solvent was used as a negative control. TM extract showed excellent antimicrobial activity against selected food related pathogenic and putrefactive bacteria (Table. 2).

While, TEA and TA were not shown significant zone of inhibition (ZOI) (data not showed). The results against Gram positive bacterial demonstrated that ZOI observed in range of between 14.33±0.58 mm to 16.67±0.58 mm, with MIC value from 3.0 mg/ml to 10.0 mg/ml for TM extract and Ampicillin showed ZOI were observed from 13.33±0.58 mm to 22.33±1.53 mm, among of them *B. cereus* ATCC13061 was more sensitive. The Gram negative bacteria showed ZOI range from 12.33±0.58 mm to 17.33±0.58 mm, with MIC value varies 5.0 mg/ml to 13.0 mg/ml. Within Gram negative bacteria *P. vulgaris* KCTC2512 showed more sensitivity to TM extract. Surprisingly, *P. aeruginosa* KCTC2004 and *S. marcescens* KCTC42171 were showed resistance to Ampicillin at 100 µg/ml but sensitive to TM extract. DMSO was not showed ZOI. Our finding as an antibacterial activity of TM against food related pathogenic and putrefactive bacteria has not been reported previously. Different extract of *C. sinensis* black tea having antimicrobial activity was reported against Gram positive, Gram negative and fungi<sup>12, 27</sup> among all food related pathogenic and putrefactive bacteria has not been reported yet.

## CONCLUSIONS

In conclusion, the preliminary phytochemical analysis for commercially available *C. sinensis* black tea revealed the presence of flavonoids, triterpenes, tannins, lipid and reducing sugar. Its antioxidant activity was confirmed through DPPH free radical scavenging assay, Reducing power assay and Total antioxidant assay. Also antibacterial activity was observed against food related pathogenic and putrefactive bacteria. The results suggest the methanol extract of *C. sinensis* black tea (TM) would be very promising natural antioxidant as well as alternative antibacterial agent in therapeutics and food industry.

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